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Relationships Between Effects of Smoking, Gender, and Alcohol Dependence on Platelet Monoamine Oxidase-B: Activity, Affinity Labeling, and Protein Measurements

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Background: Many studies have reported apparent associations between platelet monoamine oxidase (MAO) activity and susceptibility to alcoholism and other psychiatric conditions. Alcohol-dependent individuals generally exhibit lower platelet MAO activity compared with controls, and on this basis, platelet MAO has been proposed as a potential genetic marker for predisposition to alcoholism. However, several lines of evidence also suggest that MAO activity is reduced in both the brain and platelets of smokers. Many alcohol-dependent individuals are also tobacco users, and few studies have attempted to dissociate the effect of alcohol and tobacco use on MAO activity.

Methods: Platelet MAO-B activity in 629 subjects recruited as part of the WHO/ISBRA Study of State and Trait Markers of Alcohol Use and Dependence was assayed by using a high throughput fluorescence assay. Platelet MAO-B protein concentrations were measured by analysis of immunoblots probed with a polyclonal antibody selective for MAO. Quantitative measurements of affinity labeling of platelet MAO were made by using the selective MAO-B catalytic site antagonist [^{3}H]Ro 19-6327.

Results: Multiple regression analysis revealed that subjects' gender, cigarette smoking, lifetime alcohol dependence, and recruitment site each contributed independently to the variance in platelet MAO activity levels. Female subjects had significantly higher MAO activity levels than males, whereas heavy smokers had significantly lower MAO activity levels than nonsmokers. Immunoblot measurement of platelet MAO-B protein demonstrated that females had significantly higher MAO-B protein concentrations. Platelet MAO-B protein concentrations did not differ significantly between smokers and nonsmokers but were lower in subjects with a diagnosis of lifetime alcohol dependence (DSM-IV) compared with subjects who were never alcohol dependent. Measurements of affinity labeling by [^{3}H]Ro 19-6327 of platelet MAO correlated significantly with MAO activity levels (i.e., the lower MAO-B activity in smokers was mirrored by lower levels of [^{3}H]Ro 19-6327 binding).

Conclusions: In this international population, gender, cigarette smoking, lifetime history of alcohol dependence, and recruitment site were associated with lower platelet MAO activity levels. Differences in MAO activity associated with gender and lifetime alcohol dependence can be attributed largely to differences in MAO-B protein concentration, whereas those associated with smoking behavior may be the result of binding of an inhibitor contained in cigarette smoke to platelet MAO-B at catalytic site of MAO.

Key Words: Monoamine Oxidase, Smoking, Alcohol Dependence, Amplex Red, Ro 19-6327; Immunoblot; Affinity Label.

MONOAMINE OXIDASE (MAO; EC 1.4.3.4) is a flavin-containing enzyme that exists in two forms (MAO-A and MAO-B). MAO-A and MAO-B are products of separate genes that reside on the X chromosome (Bach et al., 1988; Berry et al., 1994). The isoforms of MAO can be characterized by their substrate and inhibitor specificities (Donnelly and Murphy 1977). Both MAO-A and MAO-B are present in the brain and play a significant role in monoamine metabolism and in modulation of monoamine neurotransmission (Riederer et al., 1987). MAO-B also is found in platelets, and the platelet MAO activity has been shown to be primarily or exclusively MAO-B (Denney et al., 1982; Donnelly and Murphy, 1977). The high correlation between brain and platelet MAO-B activity (Bench et al., 1991) has prompted studies that examined platelet MAO as a surrogate for brain MAO-B in individuals diagnosed with various psychiatric and neurological conditions.

These studies have demonstrated an association of low platelet MAO-B activity with the presence of certain personality characteristics (impulsiveness, risk-taking behaviors, aggressiveness), and lower levels of MAO-B in plate-

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lets also have been associated with the predisposition to drug abuse (particularly alcohol dependence; for a review, see Shih et al., 1999). On the basis of these studies, it has been postulated that individuals with low platelet MAO-B activity levels are more "vulnerable" to drug-taking behavior and that a low level of platelet MAO activity therefore may be a marker for predisposition to drug abuse and alcoholism. However, not all studies, including our own (Tabakoff et al., 1988), have found lower platelet MAO activity in alcohol-dependent individuals compared with age and gender-matched controls (Brown, 1977; Giller and Hall, 1983; Takahashi et al., 1976), and a substantial amount of work has been devoted to determining the reasons for these discrepancies (Farren et al., 1998). Issues investigated in relation to alcoholism and MAO activity have included the duration of abstinence from alcohol, race, gender, association of low MAO activity with different subtypes of alcoholism, the influence of other psychopathologies, the personality traits of alcohol-dependent individuals and, in multiple-center studies, the influence of the site at which platelets were collected (Anthenelli et al., 1998; Whitfield et al., 2000).

When we examine many of the studies on alcohol-dependent individuals, it becomes apparent that most alcohol-dependent individuals recruited into the studies were also tobacco users and that the use of tobacco use was not adequately controlled in the data analysis (Farren et al., 1998). The lower MAO-B activity in platelets (Oreland et al., 1981) of cigarette smokers has been a consistent finding in many studies (see Berlin and Anthenelli, 2001, for review). The implications of lower platelet MAO activity in smokers are amplified by investigations that used brain positron emission tomography imaging. These studies have demonstrated as much as 40% lower MAO-A and MAO-B activities in the brains of smokers compared with nonsmokers (Fowler et al., 1996a,b, 1998). More recent studies have suggested further that differences in platelet MAO activity between control and alcoholic individuals can be attributed to their smoking behavior (Anthenelli et al., 1998; Whitfield et al., 2000). However, multivariate analysis of large populations should be used to analyze simultaneously the influence of a number of variables postulated to influence MAO-B activity. Additionally, no evidence currently exists as to whether lower MAO-B activity in platelets of well-characterized subjects is a product of quantitative or qualitative differences in MAO protein.

The WHO/ISBRA Study of State and Trait Markers of Alcohol Use and Dependence was established in 1988 to assess, in a multiple-center trial, markers of recent alcohol use (state markers) and also trait markers of predisposition to alcohol dependence (Menninger et al., 2000). The extensive characterization of this subject population allowed us to examine the association of not only alcohol dependence but also age, race, gender, smoking, drug abuse, and psychiatric conditions with platelet MAO activity. By using a high throughput fluorescence assay for MAO activity, we have measured the MAO activities in platelet membranes obtained from blood samples of 629 subjects recruited into the WHO/ISBRA study and identified those variables that contributed to differences in platelet MAO-B activity among these subjects. In separate subsets of these samples, we used immunoblotting and affinity labeling methodology to assess changes in platelet MAO-B protein associated with changes in platelet MAO activity.

Table 1. Characteristics of Subjects From the WHO/ISBRA Collaborative Study

	n	%
Recruitment site		
Helsinki, Finland	76	12
Montreal, Canada	245	39
São Paulo, Brazil	145	23
Sydney, Australia	163	26
Gender (male/female)	445/184	71/29
Race		
White	521	83
Black	38	6
Asian or Indian	39	6
Other	29	5
Drinkers*	466	74
Lifetime alcohol dependence‡		
Never-dependent	295	47
Dependent	334	53
Family history of alcoholism¶	350	56
Lifetime marijuana abuse‡	48	8
Lifetime cocaine abuse‡	50	8
Major depression‡	110	17
Antisocial personality disorder‡	132	21
Conduct disorder‡	59	9
Smoking status**		
Nonsmoker	211	33
Ex-smoker	117	19
Current smoker	301	48
Age (years)	38.1 ± 12.1	Range 18–68
Total body water (liters)	38.9 ± 7.0	Range 21–58

* "Drinkers" consumed at least 15 g of ethanol in the month before blood sampling; ‡DSM-IV criteria; ¶1st or 2nd degree relatives; **Ex-smoker defined as not smoking in the last 30 day and current smoker defined as smoking at least 1 cigarette in the last 30 days.

METHODS

Subjects, Ascertainment, and Blood Samples

The subjects involved in the WHO/ISBRA project who were included in the current study were recruited in Australia (Sydney), Brazil (São Paolo), Canada (Montreal), and Finland (Helsinki). Subjects include males and females of various ages and different ethnic backgrounds (Table 1). After an initial screening and after obtaining informed consent, subjects completed the WHO/ISBRA Interview Schedule. The WHO/ISBRA Interview Schedule was adapted from the Alcohol Use Disorders and Associated Disabilities Interview Schedule developed by the NIAAA (Grant and Harford, 1990; Grant et al., 1995). It gathers a range of data that include sociodemographic details, weight and height, frequency and quantity of beverage-specific alcohol consumption during the past 30 days, symptoms of alcohol use disorders, whole-life and past 30-day drug use (prescription, over-the-counter, and illicit), history of serious illness, and smoking behavior during the past 30 days, which allow for diagnoses of alcohol, cannabis and cocaine abuse and dependence, major depressive disorder, conduct disorder, and antisocial personality disorder defined in terms of DSM-IV.

During the same visit, whole blood (30 cc) was obtained from an antecubital vein for preparation of plasma, platelets, erythrocytes, and

lymphocytes. At each site, the blood samples were collected at the time of the interview in vacutainer tubes that contained 0.10 ml of 15% EDTA solution. Platelets were isolated by using a modified method of Corash (1980). Platelet pellets were frozen and stored at −70°C until the time of assay (for more detail, see Tabakoff et al., 2002).

Platelet MAO Assays

Platelet Preparation. To standardize each assay, a platelet preparation was prepared from platelets obtained by platelet phoresis at a local blood bank. These platelets were resuspended in 100 mM potassium phosphate buffer and divided into 1 ml aliquots. The aliquots were pelleted by centrifugation (12,000 × *g*, 10 min at 4°C) and frozen as pellets that contained approximately 3 mg of protein each. Platelet membranes from this standard preparation and each subject's platelets were prepared from the frozen platelet pellets by thawing, suspension, and sonication in 1 ml of 100 mM potassium phosphate buffer (pH 7.4) followed by centrifugation at 38,000 × *g* for 10 min at 4°C. This procedure was repeated twice and the final platelet membrane pellet was resuspended in 700 μl of potassium phosphate buffer (100 mM). After protein determination (BCA assay; Pierce, Rockford, IL), platelet membranes were diluted to approximately 0.3 mg/ml in 100 mM phosphate buffer for platelet MAO activity assays. Remaining platelet membranes were pelleted by centrifugation and frozen at −70°C until used in the affinity labeling or MAO immunoblotting experiments described subsequently.

Amplex Red Fluorescence Assay for Platelet MAO Activity. Platelet MAO activity was measured by a modification of the method of Zhou and Panchuk-Voloshina (1997), which measures the amount of the coproduct, hydrogen peroxide (H_2O_2), produced during the deamination reaction. In the presence of excess N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red; Molecular Probes, Eugene, OR) and horse radish peroxidase (Sigma-Aldrich, Evanston, IL), each molecule of peroxide produced converts one molecule of Amplex Red into the stable fluorescent product resorufin. With a multichannel pipettor, wells of a 96-well microplate are filled with, sequentially, 50 μl of the substrate β-phenylethylamine, which is prepared in phosphate buffer to achieve a final concentration of 0.75, 1.5, 3.0, 6.0, or 12.0 μM, and 50 μl of a 3× reaction mixture that contains 600 μM Amplex Red (Molecular Probes, Eugene, OR) and 3 units/ml horse radish peroxidase prepared in phosphate buffer. The reaction was initiated by addition of 50 μl of platelet membranes in 100 mM phosphate buffer (pH 7.4). The plate was incubated at 37°C and fluorescence then was measured at 5 min intervals by using a fluorescent plate reader (HTS7000 BioAssay reader; Perkin-Elmer) with excitation and emission filters at 560 nm and 590 nm, respectively. All samples and standards were run in duplicate. A standard curve was generated by using serial dilutions of H_2O_2 (36 μM to 2.3 nM). The amount of MAO activity in each sample was calculated from the H_2O_2 standards by using nonlinear curve fitting (SigmaPlot, SPSS Inc., Chicago, IL). The standard platelet preparation was assayed with each group of subject samples. The interassay variability was normalized based on the activity of the standard platelet preparation.

MAO Immunoblotting. A subset of sample platelet membranes was immunoprobed for MAO protein by a modification of the method described by Gargalidis-Moudanos et al. (1997). Pelleted platelet membranes were solubilized in 100 μl of 2% sodium dodecyl sulfate (SDS) by boiling for 3 min. Protein concentrations were determined followed by addition of 25 μl of 5× SDS sample buffer (0.3125 M Tris, 10% SDS, 750 mM dithiothreitol, 7.5 M urea, 50% glycerol, 0.05% bromphenol blue) and boiling to denature proteins. Protein (5 μg per well) was loaded onto 8% SDS-PAGE gels, and proteins were separated and transferred to nitrocellulose membranes by standard SDS-PAGE procedures. Blots were blocked in 5% nonfat dry milk in Tris-buffered saline (TBS), pH 7.5, and 0.1% Tween 20 (NFDM-TBST), washed twice in washing buffer (TBS and 0.05% Tween 20, TBST-0.05), and then incubated for 1 hr at room temperature with rabbit polyclonal antisera (1:1000 dilution in 5% NFDM-TBST). The polyclonal antisera, kindly provided by Dr. Angelo Parini (INSERM, France), were obtained from rabbits immunized with a peptide sequence common to both MAO-A and MAO-B (Gargalidis-Moudanos et al., 1997). After incubation, blots were washed twice and then incubated with horse radish-peroxidase-conjugated antirabbit immunoglobulin G (Bio-Rad, Hercules, CA) diluted 1:10,000 in NFDM-TBST. Bound antibodies were detected by using enhanced chemiluminescence (Renaissance, Dupont-NEN, Boston, MA) and exposure to Kodak X-Omat film. The optical density of immunoreactive bands was determined and analyzed by using Molecular Analyst software (Bio-Rad) with local background subtraction. In each experiment, a standard curve was generated by using increasing protein amounts of the standard platelet preparation (1–20 μg), and data were fitted to a three-parameter Hill equation by using nonlinear curve fitting (SigmaPlot). Quantification of sample band intensities was performed by using the adjusted optical density of the sample bands relative to the standard curve. Results were expressed as microgram equivalents of the MAO immunoreactivity (relative to the standard platelet preparation) per microgram of total protein.

Affinity Labeling. A random subset of subject platelet membranes were affinity labeled with [^{3}H]Ro 19-6327, a selective, high-affinity MAO-B inhibitor (Cesura et al., 1988). Platelet membranes were resuspended in 100 mM potassium phosphate buffer (pH 7.4) as described previously for MAO activity measurements. The final resuspension was divided evenly between two microcentrifuge tubes (approximately 100 μg of total platelet protein per tube). The irreversible MAO-B inhibitor l-deprenyl (200 μM, final concentration in one tube) or an equivalent volume of phosphate buffer (other tube) was added, and both tubes were incubated for 15 min at 37°C. Platelet membranes then were incubated for 1.5 hr at 20°C in the presence of 100 nM [^{3}H]Ro 19-6327 (Amersham Pharmacia Biotech, Piscataway, NJ). Sodium cyanotetrahydroborate ($NaBH_3CN$, 2 mg/ml, final concentration) was added and the pH rapidly adjusted to pH 4.5 with acetic acid. After an additional 20 min incubation at room temperature, 100 μg of bovine serum albumin was added to each tube, and total protein was precipitated by addition of ice-cold 10% trichloroacetic acid. The tubes were centrifuged at 13,000 rpm for 10 min. The supernatant was aspirated and the pellets were washed twice more with ice-cold 5% trichloroacetic acid. After the final wash, the pellets were solubilized by addition of 50 μl of 1 N NaOH and overnight incubation at room temperature. The samples then were diluted with 450 μl of 100 mM phosphate buffer (pH 7.4) and transferred to scintillation minivials followed by the addition of 4 ml of UltimaGold scintillation cocktail (Packard Bioscience, Groningen, Netherlands). Radioactivity was counted on a Beckman TA6100 scintillation spectrometer. The affinity labeling of MAO-B protein with [^{3}H]Ro 19-6327 was calculated by subtracting the amount of radioactivity measured in the presence of l-deprenyl from the amount of radioactivity measured in its absence. The molar amount of [^{3}H]Ro 19-6327 irreversibly bound was calculated from the specific activity of the ligand (16 Ci/mmol) and expressed as fmol/μg protein.

Data Analysis

General. MAO activity data for platelet standards and subject samples were fit to a three-parameter Hill equation to obtain estimates of affinity (K_m) and maximal velocity (V_{max}). Preliminary analyses indicated that lower MAO activity in subject platelet samples in this database were due to differences in the V_{max} without changes in K_m. This finding has been reported in other similar studies of platelet MAO activity (Anthenelli et al., 1995). The V_{max} values for MAO were significantly and positively skewed ($p < 0.001$; Kologorov-Smirnov test). Consequently, bivariate and multiple regression analyses were performed on the log-transformed V_{max} data of all subjects.

Statistics. Multiple regression analysis was used to evaluate the relationship of 14 independent variables, with platelet MAO-B activity (V_{max}) as the dependent variable. The variables chosen for testing in the models were based on our previous work (Anthenelli et al., 1995; Tabakoff et al., 1988) and that of others (Anthenelli et al., 1998; Whitfield et al., 2000) that has examined the association of alcohol dependence with platelet MAO activity. Table 1 lists the variables included in our analysis. Effects of dropping variables from the regression model on the overall goodness-of-fit were assessed by backward stepwise regression (SPSS Inc.). By using

Table 2. Multiple Regression Model Predicting Variance in Platelet MAO Activity (log V_{max})

	β	95% CI		p
Recruitment site	−0.044	−0.058	−0.031	<0.001
Gender	0.141	0.081	0.201	<0.001
Smoking status (compared with nonsmokers)	−0.046	−0.068	−0.024	<0.001
Alcohol dependence	−0.035	−0.073	0.004	0.020
Total body water	0.004	0.000	0.002	0.066

Number of observations, 626; $R^2 = 0.176$. The final regression model was obtained by backward stepwise regression. Independent variables listed were significantly associated with platelet MAO activity (log-likelihood ratio test) at the indicated level of significance in the model. The regression coefficient (β) and the 95% confidence interval (CI) are given. Although not statistically significant, total body water was determined to be confounded with gender and therefore included in the final model.

the log-likelihood ratio test, each independent variable was removed sequentially if it was not significant at the $\alpha = 0.05$ level. As variables were removed, their potential as confounding variables was assessed by calculating a change in the coefficients of the other variables in the model. Variables that produced changes greater than 15% were considered confounders and were left in the model. This process was repeated until covariates could no longer be eliminated, leaving a primary main effects model. Nonlinear terms were either collapsed into meaningful categories or mathematically transformed. A series of ANOVAs were performed to examine the major factors influencing MAO activity as determined by the regression analysis by using SPSS (version 9.0). When statistical significance was evident, post hoc analyses were performed by using Tukey's honestly significant difference test for multiple comparisons.

RESULTS

Platelet MAO-B activity measurements were completed for 646 subjects by using the Amplex Red fluorescence assay of MAO-B activity. Platelet log(V_{max}) values for MAO activity that were more than 2 standard deviations greater than the sample average were excluded from analysis ($n = 17$). The preponderance (83%) of the remaining subjects were white and well educated, with 78% of the subjects having completed high school or having attended a university as an undergraduate or postgraduate. The subjects covered a significant age range, with 44% of the subjects being between the ages of 18 and 35, 45% being between 35 and 55, and the rest being 55 years or older. Female subjects were recruited only at the Montreal and São Paolo sites, and the total study population was composed of 71% males and 29% females. Three hundred and thirty-four subjects (53%) met the DSM-IV criteria (Grant et al., 1995) for lifetime alcohol dependence. The remaining subjects were nondrinkers or drinkers who had never met the diagnostic criteria for alcohol dependence. Recent (last 30 days) alcohol intake was significantly greater in dependent individuals (2302 ± 141 g) compared with the 132 drinkers in the never-dependent group (736 ± 100 g; $p < 0.001$, independent t test). Thirty-three percent of the subjects had never smoked, and 19% were ex-smokers (no use of tobacco products in the last 30 days). Forty-eight percent of the study subjects were current smokers whose use ranged from 1 to 1800 cigarettes smoked in the month before recruitment. A small number (5%) of the current smokers used other tobacco products (pipe or cigars) as well.

Table 2 shows the results of the multiple regression analysis with platelet MAO activity as the dependent variable. Due to the listwise deletion of variables, the final main effects multiple regression model for the platelet MAO activity represented 626 of the original 629 subjects. Subjects' gender, smoking status, lifetime history of alcohol dependence, and the recruitment site at which platelet samples were collected significantly predicted 18% of the variance ($R^2 = 0.18$, $p < 0.001$) in platelet MAO activity levels. Although not statistically significant, total body water (TBW) influenced the relationship between gender and MAO activity. As TBW increased, MAO activity decreased. When this variable was removed from the model, the coefficient for gender changed by 18%. This association is likely due to the fact that gender is a determinant of TBW, with males having significantly higher TBW than females (42.4 ± 0.2 liters and 30.5 ± 0.3 liters, respectively; $p < 0.001$, independent t test). Major depression, conduct disorder, antisocial personality disorder, lifetime marijuana abuse, and lifetime cocaine abuse did not explain any additional variance in platelet MAO-B activity. Nor was platelet MAO activity influenced by the subjects' race, age, or family history of alcohol dependence. The regression analysis also failed to reveal any significant interactions between variables in the final model, which indicated that gender, smoking status, lifetime history of alcohol dependence, and the recruitment site each accounted for a portion of the variance independent of each other.

The regression results are supported by univariate comparisons between groups. Thus, when the number of cigarettes smoked in the last 30 days was used to categorize current smokers (<300 cigarettes/month, 300–600 cigarettes/month, >600 cigarettes/month), increasing cigarette use was associated with progressively lower MAO activity, which reached statistical significance when smoking exceeded 300 cigarettes/month compared with non- or ex-smokers (Fig. 1). MAO activities for individuals smoking <300 cigarettes/month were not different from non- or ex-smokers. This dose-response relationship was exhibited by both genders, although females had significantly higher MAO-B activity compared with males, regardless of smoking status.

The average MAO activities differed as a function of the recruitment sites at which the platelets were collected, with platelet MAO activities highest in the Helsinki samples and lowest in the Sydney samples. Despite these differences,

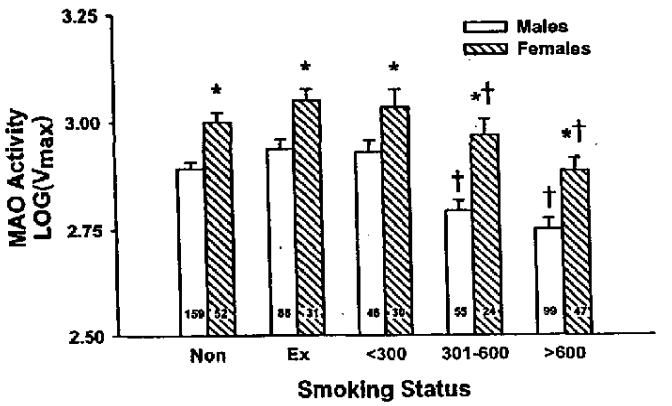

Fig. 1. Smoking and gender effects on platelet MAO activity. Platelet MAO activity is expressed as the mean ± SE of the log-transformed V_{max} values for nonsmokers (Non), ex-smokers (Ex), and current smokers. For current smokers, the number of cigarettes smoked during the previous month is shown. *Significantly different from males [$F(1,619) = 43.7$, $p < 0.001$]; †significantly different from non-, ex-, and light smokers [$F(4,619) = 13.5$, $p < 0.001$].

the effect of smoking was present at all sites (data not shown).

Immunoblotting

The polyclonal antibody used in these experiments was raised against a polypeptide common to both MAO-A and MAO-B and therefore can recognize both MAO isoforms (Gargalidis-Moudanos et al., 1997). In the standard platelet preparation, this antibody detected a single polypeptide with apparent molecular mass of ~59 kDa (data not shown). This value is in good agreement with the molecular weight predicted from the amino acid sequence of MAO-B (Shih et al., 1999).

A subset of platelet samples from 58 subjects was selected from the entire dataset for measurement of MAO-B protein concentration. The samples were chosen to be representative of the MAO-B V_{max} values in the entire dataset with regard to smoking status and gender. Platelet MAO-B activity in this subset (Fig. 2A) mirrors the smoking and gender differences seen in the entire subject population; that is, platelet MAO-B activity was significantly lower in the smokers compared with nonsmokers and ex-smokers in both males and females, and MAO-B activity was significantly higher in females regardless of smoking status. The quantitation of immunoblots (Fig. 2B) demonstrated that MAO-B protein concentration from platelets from female subjects was significantly higher than males. However, MAO-B protein concentration in platelets of male and female smokers did not differ significantly from male and female non- or ex-smokers (Fig. 2B). When the catalytic activity was expressed as "specific activity" (fmol product/μg equivalent MAO-B/min, Fig. 2C), the gender difference was no longer evident. Smokers' MAO-B specific activity was lower than nonsmokers' and ex-smokers' (Fig. 2C). When the concentration of MAO-B protein was

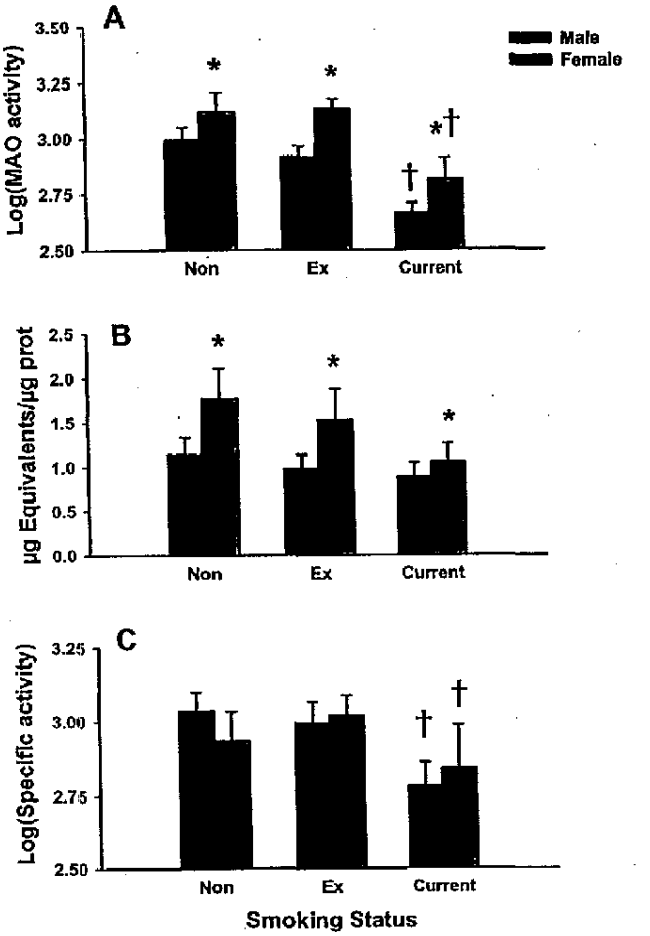

Fig. 2. Measurements of platelet MAO-B activity, protein concentration, and specific activity in a representative subset of 58 subjects from the WHO/ISBRA dataset. (A) MAO-B activity. Data are the mean ± SE of the log-transformed V_{max} values. *Significantly different from males [$F(1,52) = 10.3$, $p < 0.01$]; †significantly different from nonsmokers (Non) and ex-smokers [Ex; $F(2,52) = 15.3$, $p < 0.001$] with no interaction between smoking and gender [$F(2,52) = 0.3$, $p = 0.75$]. (B) MAO-B protein concentration in the same platelet samples. Data are the mean ± SE of the μg equivalents/μg total protein of the standard platelet preparation. *Significantly different from males [$F(1,52) = 5.3$, $p < 0.05$]. Current smokers were not significantly different from non- or ex-smokers [$F(2,52) = 2.0$, $p = 0.14$] with no interaction between smoking and gender [$F(1,52) = 0.5$, $p = 0.60$]. (C) MAO-B-specific activity. Data are the mean ± SE of the log-transformed values as calculated as described in the text. †Different from non- and ex-smokers [$F(2,52) = 2.9$, $p = 0.06$]. Females did not differ from males [$F1,52) = 0.01$, $p = 0.933$], and there was no interaction between these two variables [$F(1,52) = 0.48$, $p = 0.62$].

plotted against platelet MAO activity, a positive linear relationship was observed in the platelet membranes from non- and ex-smokers ($R = 0.62$, $p < 0.001$) but not in platelet membranes from current smokers ($R = 0.07$, $p = 0.84$; Fig. 3A).

The subjects then were grouped according to the presence or absence of DSM-IV criteria of lifetime alcohol dependence. To avoid the confound of smoking on platelet MAO activity measures, we assessed the influence of alcohol dependence only in non- and ex-smokers. Figure 4A shows that platelet MAO activity in subjects who met

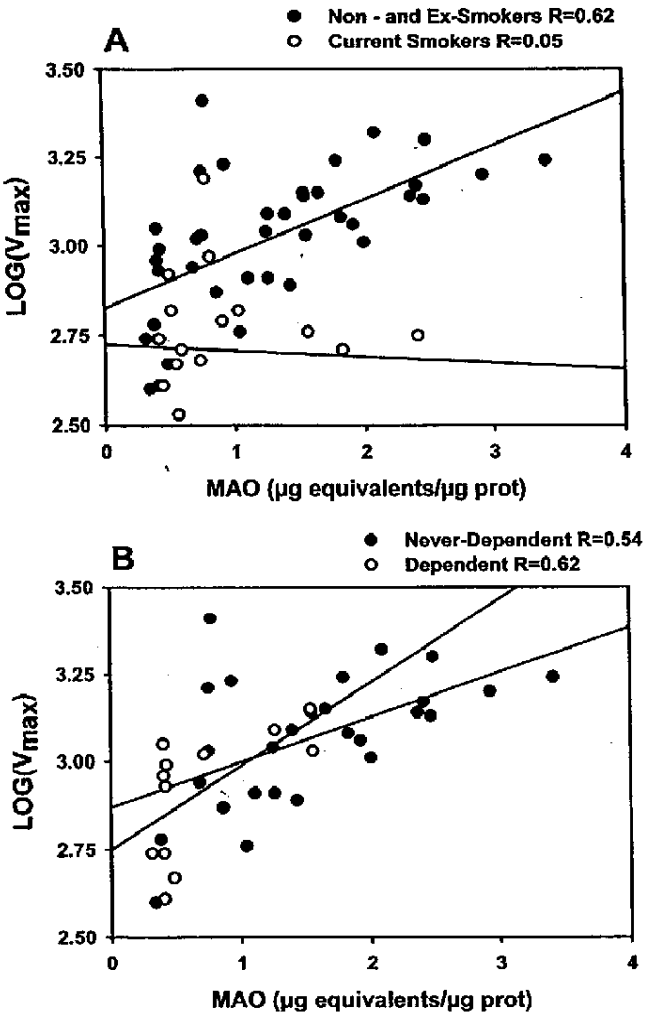

Fig. 3. Correlations between platelet MAO-B activity and platelet MAO-B protein concentration in the subjects depicted in Figs. 2 and 4. (A) Platelet MAO activity was significantly correlated with MAO-B protein concentration in non- and ex-smokers ($R = 0.62$, $p < 0.001$) but not in current smokers ($R = 0.05$). (B) Platelet MAO activity was correlated significantly with MAO-B protein concentration in both never-dependent ($R = 0.54$, $p < 0.001$) and dependent subjects ($R = 0.62$, $p < 0.001$).

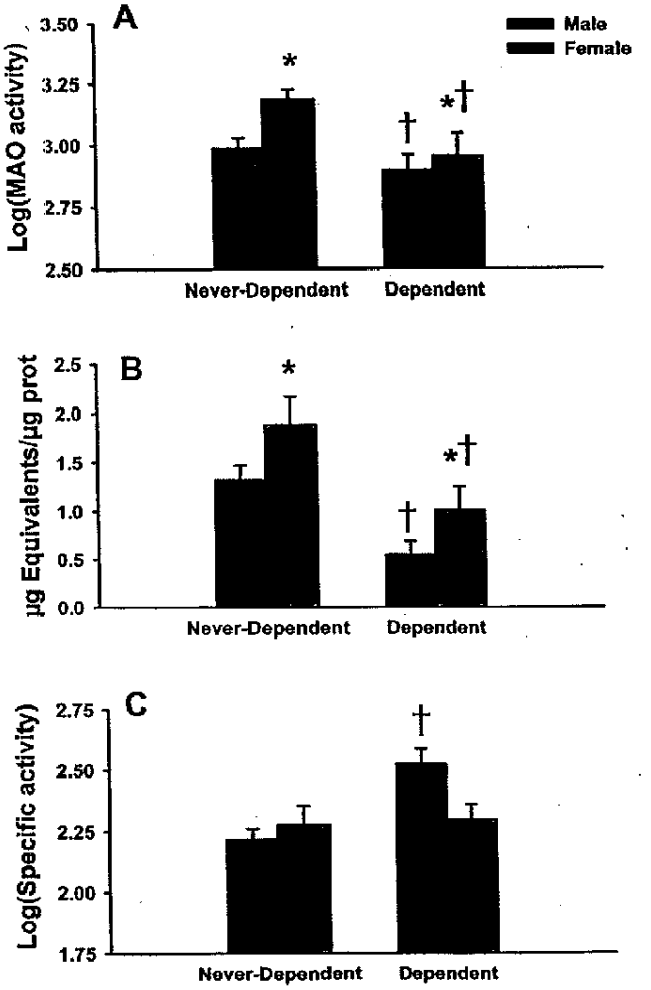

Fig. 4. Measurements of platelet MAO-B activity, protein concentration, and specific activity in non- and ex-smokers from the subset sample shown in Fig. 2 ($n = 39$) grouped by DSM-IV criteria for lifetime alcohol dependence. (A) *Significantly different from males [$F(1,35) = 4.3$, $p < 0.05$]; †significantly different from never-dependent subjects [$F(1,35) = 6.9$, $p < 0.02$] with no interaction between dependence and gender [$F(1,35) = 1.4$, $p = 0.251$]. (B) *Significantly different from males [$F1,35 = 4.3$, $p < 0.05$]; †significantly different from never-dependent subjects [$F(1,35) = 11.3$, $p < 0.005$] with no interaction between gender and alcohol dependence [$F(1,35) = 0.04$, $p = 0.851$]. (C) Analysis of specific activity indicated a significant effect of alcohol dependence [$F(1,35) = 5.0$, $p < 0.05$], no significant main effect of gender [$F1,35) = 1.4$, $p = 0.252$] with a significant interaction between these variables [$F(1,35) = 4.0$, $p < 0.05$]. †Significantly different from male never-dependent subjects ($p < 0.001$).

DSM-IV criteria for lifetime history of alcohol dependence was significantly less than that exhibited by never-dependent subjects. Females in this subset of subjects demonstrated higher platelet MAO activity, and this difference was not influenced by a lifetime history of alcohol dependence. Platelet MAO-B protein concentration (Fig. 4B) from female subjects was, again, higher than males. However, the diagnosis of alcohol dependence was associated with a significantly lower platelet MAO-B protein concentration. Although the specific activity of never-dependent females and males did not differ, subjects with lifetime alcohol dependence displayed significantly higher platelet MAO specific activity that could be attributed to particularly low MAO protein levels in males with lifetime history of alcohol dependence. When the concentration of MAO-B protein was plotted against platelet MAO activity, a positive correlation was observed in the platelet membranes from both never-dependent ($R = 0.54$, $p < 0.001$) and dependent subjects ($R = 0.62$, $p < 0.001$; Fig. 3B).

Affinity Labeling

Cesura et al. (1988) previously reported that, under the conditions used in this study, [^{3}H]Ro 19-6327 selectively and irreversibly binds to MAO-B in platelets and that preincubation of membranes with unlabeled selective, irreversible MAO-B inhibitors, like l-deprenyl, prevents the

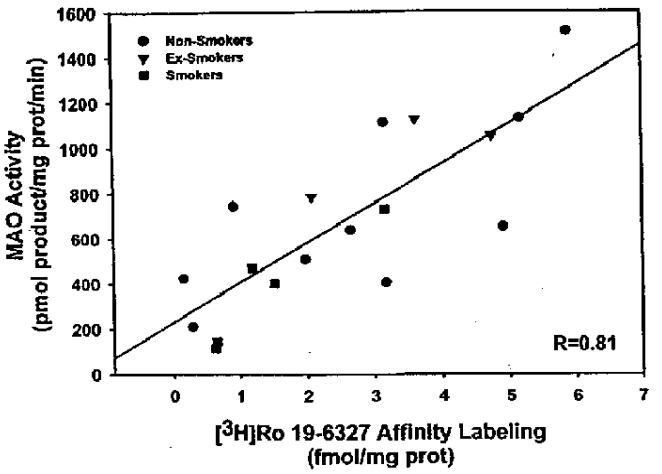

Fig. 5. Correlation between [^{3}H]Ro 19-6327 affinity-labeling and MAO-B activity in platelet membranes of subjects classified as nonsmokers, ex-smokers, or current smokers. Platelet MAO activity was significantly correlated with the amount of affinity-labeling ($R = 0.81$, $p < 0.001$).

[^{3}H]Ro 19-6327 affinity-labeling. When affinity-labeled platelet membranes were subjected to SDS-PAGE and immunoblotted with the polyclonal antibody to MAO, all the radioactivity in the solubilized sample comigrated with the 59 kDa immunoreactive polypeptide (data not shown). We found that the amount of [^{3}H]Ro 19-6327 irreversibly bound to platelet membranes from non-, ex-, and current smokers was significantly correlated with the amount of MAO activity previously measured in these membranes (Fig. 5; $R = 0.81$, $p < 0.001$).

DISCUSSION

The present results demonstrate that in a large international population that included individuals of both genders and covered significant ranges of both age and alcohol consumption, four factors (i.e., gender, recruitment site, alcohol dependence, and cigarette smoking) predicted a significant amount of the variance in platelet MAO activity. Family history of alcoholism, other substance abuse (marijuana, cocaine), or other psychiatric conditions (major depression, antisocial personality disorder, and conduct disorder) did not account for any additional variance. Other variables tested that did not significantly contribute to the final regression model were age, race, and volume of alcohol consumed over the last 30 days.

Although age has, in the past, been associated with increases in MAO activity, such increases were seen in populations of >60 years of age (Veral et al., 1997). In our study, few (seven) individuals >60 years of age were included. Our data also suggest that the use of alcohol per se does not account for the association of alcohol dependence with lower platelet MAO activity. Differences in platelet MAO activity in samples from different recruitment sites have been reported in other multicenter studies (Anthenelli et al., 1998; Whitfield et al., 2000). Although subjects were recruited in four different countries in our study, the vast majority of subjects were white and the effect of race was not significant. The lack of interaction of the recruitment site variable in the current study with any of the other variables in the regression analysis indicates that the recruitment site variable was not confounded by an unbalanced representation of smoking subjects within a recruitment site. Despite differences in the absolute values of platelet MAO activity that existed between sites, MAO activity in samples from current smokers was always lower than activity in samples from non- and ex-smokers (data not shown). Contrary to other recent reports (Anthenelli et al., 1998; Whitfield et al., 2000), we found that after the variations in smoking, gender, and recruitment site were taken into account, the diagnosis of lifetime alcohol dependence was still significantly associated with low MAO activity. Thus, unlike the previous reports, we cannot conclude that lower platelet MAO-B activity in alcohol-dependent subjects is merely a consequence of heavy smoking behavior exhibited by these individuals.

However, to our knowledge, ours is the first study to examine the relationship between differences in platelet MAO-B protein concentration and platelet MAO activity associated with gender, smoking, and alcohol dependence. Although our studies could only be performed on a representative subset of the entire population, the data obtained offer important insights into some of the previous inconsistencies reported in the literature. Our data support previous reports (Anthenelli et al., 1995; Whitfield et al., 2000) that females have higher platelet MAO activity than males and also offer a possible explanation for the gender differences in platelet MAO activity observed by others. We found that platelet membranes from women contained a significantly greater concentration of MAO-B protein than platelet membranes from men, regardless of smoking status (Fig. 2B) or history of alcohol dependence (Fig. 4B). When platelet MAO-B activity was adjusted for the level of MAO-B protein (i.e., specific activity was calculated), significant gender differences were no longer apparent, which suggested that different levels of MAO-B protein can explain the MAO activity differences between males and females.

Lower MAO activity in the platelets of human smokers, compared with nonsmoking controls, has been reported several times—14–53% (Berlin et al., 1995b, 2000; Rose et al., 2001; Saccone et al., 1999) and 25% in this study—and platelet MAO activity values in relation to smoking follow a general dose-response relationship with lower values being evident as the average number of cigarettes smoked increases (Saccone et al., 1999; Whitfield et al., 2000; Fig. 1). These reports suggest that lower platelet MAO-B activity in smokers results from a pharmacological inhibition of platelet MAO-B. Our findings that platelet MAO-B protein concentration did not differ on the basis of smoking

status (Fig. 2B) and that the calculation of specific activity did not eliminate the inhibitory effect of cigarette smoking on platelet MAO activity (Fig. 2C) are consistent with this hypothesis. MAO-B inhibition observed in smokers is not due to nicotine, because in vitro studies have shown that nicotine concentrations 2000 times higher than levels attained in the blood of heavy smokers are required before significant platelet MAO inhibition is achieved (Oreland et al., 1981). Compounds that reversibly inhibit MAO activity have been identified in tobacco extracts (Khalil et al., 2001; Mendez-Alvarez et al., 1997) and, in the presence of the formaldehyde and cyanide also found in cigarette smoke, may form adducts with the reactive amino groups of the MAO protein (Boulton et al., 1988), which possibly would decrease its catalytic activity. The ability to measure, in our studies, reduced MAO-B activity in well-washed platelet membranes (see "Methods") obtained from smokers and the slow "normalization" in MAO activity reported in platelets (Berlin et al., 1995a) after smoking cessation suggests that the inhibition of MAO by the products of cigarette smoke may be irreversible. Our finding that MAO-B activity is correlated positively with MAO-B protein concentration in the platelet membranes of non- and ex-smokers but not current smokers (Fig. 3A) is compatible with the hypothesis that some proportion of platelet MAO-B protein in smokers is bound irreversibly by the components of cigarette smoke and that the normalization of platelet MAO activity may require the generation of new platelets that contain uninhibited MAO-B protein.

Further evidence for an active site-directed inhibitor in tobacco smoke is apparent in our studies that measured affinity labeling by Ro 19-6327, a selective MAO-B inhibitor that binds at the substrate recognition site (Cesura et al., 1989). Under the concentration and incubation conditions used in our work, the binding of [^{3}H]Ro 19-6327 proceeds to saturation. Therefore, the amount of [^{3}H]Ro 19-6327 irreversibly bound to platelet membranes can be used as a measure of the amount of available MAO-B substrate recognition sites. A parsimonious explanation for the close correlation of [^{3}H]Ro 19-6327 affinity-labeling and MAO activity levels in platelet membranes from nonsmokers, ex-smokers, and current smokers (Fig. 5) is that Ro 19-6327 and the irreversible inhibitor contained in cigarette smoke bind to a common site, that is, the substrate binding site. Although intriguing, this proposed mechanism and site of action for the inhibition of MAO activity by cigarette smoke will need further substantiation because studies of MAO-B structure indicate that there are several regions in the MAO-B amino acid sequence whose modification can result in a significant loss of enzymatic activity (Abell and Kwan, 2001).

When grouped by alcohol dependence status, subjects who met criteria for lifetime alcohol dependence exhibited lower platelet MAO-B activity and MAO protein concentration than never-dependent subjects (Fig. 2A and 2B). Correlation analysis indicated that MAO-B activity could be predicted on the basis of the concentration of MAO-B protein concentration in the platelet membranes, regardless of the diagnosis of alcohol dependence (Fig. 4B). Surprisingly, the specific activity of alcohol-dependent subjects was found to be significantly higher than that of never-dependent subjects, primarily among the male subjects (Fig. 4C).

Although both alcohol dependence and smoking contribute to lower platelet MAO-B activity, they appear to do so by different mechanisms. Smoking produces a pharmacological inhibition of the MAO-B protein present in platelets. On the other hand, individuals with a history of lifetime alcohol dependence have lower platelet MAO-B activity as a consequence of lower MAO-B protein concentration. However, one has to be extremely cautious in interpreting our results as a demonstration that low platelet MAO activity associated with low platelet MAO-B protein concentration represents a trait marker for predisposition to alcohol dependence, because we saw no statistical relationship between low MAO activity and a family history of alcohol dependence. In addition, our findings need to be considered in the context of the nature of the cohort used in our study compared with others (Anthenelli et al., 1998; Whitfield et al., 2000). A greater proportion (53%) of our subjects met criteria for lifetime alcohol dependence compared with 34% and 21% of subjects in the studies by Anthenelli et al. (1998) and Whitfield et al. (2000), respectively. In addition, 50% of the subjects in our sample had at some time sought treatment for alcohol-related problems compared with 1% in the Whitfield et al. (2000) sample, which suggests that low platelet MAO activity (and low platelet MAO-B protein concentration) may be a marker for alcohol dependence if dependence is severe enough such that the subject seeks treatment.

In summary, our present studies demonstrate that smoking, gender, and a history of alcohol dependence had significant influence on platelet MAO-B activity in our sample of adult men and women who participated in the WHO/ISBRA study. The higher platelet MAO activity in women was associated with higher concentrations of MAO-B protein in platelets. The reduction of MAO activity by cigarette smoking may reflect a reduced substrate accessibility to the MAO catalytic site as a result of the irreversible binding of a yet-to-be-identified component of cigarette smoke. In contrast, reduced platelet MAO activity in subjects with a lifetime history of alcohol dependence may be a consequence of a reduced concentration of platelet MAO-B protein. Consideration of platelet MAO-B protein measurements in the investigation of other psychiatric and neurological conditions associated with altered platelet MAO activity may give better insights into the pharmacological and genetic bases for these associations.

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